

**ANION BINDING TO PROTEINS**

**A NUCLEAR QUADRUPOLEAR RELAZATION  
STUDY OF HALIDE NUCLEI**

Jan-Erik Norne

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UNION TYPING TO PROTECT

A VARIOUS ECONOMIC RESEARCH

STUDY OF HALLS HOUSE

ONE-STEP WORK



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ANION BINDING TO PROTEINS. A NUCLEAR QUADRUPOLEAR  
RELAXATION STUDY OF HALIDE NUCLEI.

NMR on quadrupolar nuclei has in the last ten years become a powerful method for the study of a large variety of biological systems (1). Quadrupolar halide nuclei were first used to probe macromolecular properties in studies on mercury labelled SH-groups on proteins (2). Halide ion - heavy metal interactions were known from measurements on low molecular complexes to give large effects on the relaxation of quadrupolar anions. The first studies on mercury labelled hemoglobin by Stengle and Baldeschwieler were followed by similar measurements on native metallo-proteins (3). It was of great significance when studies were presented which showed that non-metalloproteins also gave significant effects on chloride and bromide relaxation rates (4).

The present thesis gives some examples of the use of quadrupolar halide nuclei to study the interaction between anions and proteins. The purpose of this summary is to give a brief review of the use of the halide probe method for investigation of certain aspects of macromolecule chemistry. Most examples will be taken from the papers presented in the thesis.

The following papers will be treated:



- I. Chiancone, E., Norne, J-E., Forsén, S., Antonini, E. and Wyman, J.. Nuclear magnetic resonance quadrupole relaxation studies of chloride binding to human oxy- and deoxyhemoglobin. *J.Mol.Biol.* 70 675 (1972).
- II. Norne, J-E., Bull, T., Einarsson, R., Lindman, B. and Zeppezauer, M.. On the discrimination between metal-coordinative and general anion binding to proteins by  $^{35}\text{Cl}$  NMR. *Chem.Scr.* 3 142 (1973).
- III. Chiancone, E., Norne, J-E., Bonaventura, J., Bonaventura, C. and Forsén, S.. Nuclear magnetic resonance quadrupole relaxation study of chloride binding to hemoglobin Abruzzo ( $\alpha_2^A \beta_2^{143\text{His}\rightarrow\text{Arg}}$ ). *Biochim.Biophys.Acta* 336 403 (1974).
- IV. Norne, J-E., Csopak, H. and Lindman, B..  $^{35}\text{Cl}$  nuclear magnetic resonance study of zinc and phosphate binding to *E. coli* alkaline phosphatase. *Arch.Biochem.Biophys.* 162 552 (1974).
- V. Chiancone, E., Norne, J-E., Forsén, S., Brunori, M. and Antonini, E.. Nuclear magnetic resonance quadrupole relaxation studies of chloride binding to the isolated hemoglobins from trout. *Biophys.Chem.* 3 56 (1975).
- VI. Norne, J-E., Lilja, H., Lindman, B., Einarsson, R. and Zeppezauer, M..  $\text{Pt}(\text{CN})_4^{2-}$  and  $\text{Au}(\text{CN})_2^-$  : Potential general probes for anion binding sites of proteins. *Eur.J.Biochem.* 59 463 (1975).
- VII. Chiancone, E., Norne, J-E., Forsén, S., Bonaventura, J., Brunori, M., Antonini, E. and Wyman, J.. Identification of chloride-binding sites in hemoglobin by nuclear magnetic resonance quadrupole relaxation studies of hemoglobin digests. *Eur.J.Biochem.* 55 385 (1975).
- VIII. Norne, J-E., Hjalmarsson, S-G., Lindman, B. and Zeppezauer, M.. Anion binding properties of human serum albumin from halide ion quadrupole relaxation. *Biochem.* 14 3401 (1975)
- IX. Chiancone, E., Norne, J-E., Forsén, S., Mansouri, A. and Winterhalter, K.. Anion binding to proteins. NMR quadrupole relaxation study of chloride binding to various human hemoglobins. *FEBS Lett.* 63 309 (1976).



- X. Chiancone, E., Bull, T., Norne, J-E., Forsén, S. and Antonini, E..  
Studies on Erythrocrucorin. Nuclear magnetic resonance  
quadrupole relaxation study of sodium, calcium and  
chloride binding. J.Mol.Biol. 107 25 (1976).

For nuclei with spin quantum number (I) greater than 1/2 the distribution of charge over the nucleus is not spherical. The deviation from spherical distribution may be described in terms of a nuclear electric quadrupole moment (eQ). A quadrupole moment can interact with electrical field gradients and thus provide a coupling between the nucleus and its environment. In particular, fluctuating field gradients at the nucleus can be an effective mechanism for nuclear magnetic relaxation. In most systems with quadrupolar nuclei, this is the only significant relaxation mechanism. This situation often makes the interpretation of relaxation easier than for non-quadrupolar nuclei.

Hubbard (5) has derived equations for the decay of magnetization of nuclei with spin  $I = 3/2$  (e.g.  $^{35}\text{Cl}$ ,  $^{37}\text{Cl}$ ,  $^{79}\text{Br}$  and  $^{81}\text{Br}$ ) undergoing quadrupolar relaxation. The time dependence of the longitudinal magnetization should, in the general case, follow the equation:

$$M_z - M_{z\infty} = M_z^0 \left\{ 0.8e^{-a_1 t} + 0.2e^{-a_2 t} \right\}$$

and the in-phase component in the rotating frame representation should be written:

$$M_x = M_x^0 \left\{ 0.6e^{-b_1 t} + 0.4e^{-b_2 t} \right\}$$



where  $a_1$ ,  $a_2$ ,  $b_1$  and  $b_2$  are functions of the quadrupole coupling constant ( $e^2 Qq/h$ ) and the spectral densities of the fluctuating field gradients at 0,  $\omega$  and  $2\omega$  ( $\omega$  is the resonant frequency). If the correlation time for this fluctuation  $\tau_c$  is much shorter than the inverse resonant frequency,  $1/\omega$ , (i.e. extreme narrowing conditions) then  $a_1=a_2=b_1=b_2$  and the decay of both the longitudinal and transverse magnetizations can be described by a single relaxation time:

$$\frac{1}{T_1} = \frac{1}{T_2} = \frac{2\pi^2}{5} (e^2 Qq/h)^2 \tau_c$$

Halide nuclei have low intrinsic NMR sensitivity so it is not practical to observe directly the very broad signals from nuclei bound to a macromolecule. However, if the ions exchange rapidly (i.e. faster than the decay of magnetization in the bound state) between free and bound states, this exchange will provide the magnetization of the free nuclei with an efficient path to equilibrium, and the observed relaxation rate of the bulk magnetization will be a population weighted average of relaxation in the free and bound state. Binding of an aqueous halide ion to a macromolecule will slow down its tumbling motion and increase the field gradient at the nucleus. This will result in a marked increase in relaxation rate and the condition of extreme narrowing will generally not be fulfilled. If the exchange of the halide is slow compared to relaxation of the bound nucleus the contribution from the bound nuclei will be smaller. In the limit of long exchange times



the observed relaxation time is not influenced by the relaxation of the bound nucleus. Bull (6) has derived general equations for relaxation of exchanging spin  $3/2$  nuclei under non-extreme narrowing conditions. Bull has also modified these equations to apply to the halogen probe method when the deviation from extreme narrowing conditions is not too severe, ( $\omega\tau_c \leq 1.5$ ). For this case the decay of magnetization will follow a single exponential and a relaxation time can be defined. According to Bull the relaxation rates for aqueous quadrupole nuclei in equilibrium with a macromolecule should follow the equations:

$$\frac{1}{T_1} = \frac{1}{T_{1A}} + p_B \left\{ \frac{0.8}{T'_{1B} + \tau_B} + \frac{0.2}{T''_{1B} + \tau_B} \right\}$$

where  $\frac{1}{T'_{1B}} = \frac{2\pi^2}{5} \left( \frac{e^2 q Q}{h} \right)^2 \frac{\tau_c}{1 + 4\omega^2 \tau_c^2}$

and  $\frac{1}{T''_{1B}} = \frac{2\pi^2}{5} \left( \frac{e^2 q Q}{h} \right)^2 \frac{\tau_c}{1 + \omega^2 \tau_c^2}$

$$\frac{1}{T_2} = \frac{1}{T_{2A}} + p_B \left\{ \frac{0.6}{T'_{2B} + \tau_B} + \frac{0.4}{T''_{2B} + \tau_B} \right\}$$

where  $\frac{1}{T'_{2B}} = \frac{2\pi^2}{5} \left( \frac{e^2 q Q}{h} \right)^2 \left[ \tau_c + \frac{\tau_c}{1 + \omega^2 \tau_c^2} \right]$

and  $\frac{1}{T''_{2B}} = \frac{2\pi^2}{5} \left( \frac{e^2 q Q}{h} \right)^2 \left[ \frac{\tau_c}{1 + 4\omega^2 \tau_c^2} + \frac{\tau_c}{1 + \omega^2 \tau_c^2} \right]$

$p_B$  is the fraction of nuclei bound to the macromolecule.



The use of NMR of quadrupolar halide nuclei to probe macromolecular properties is not limited to the study of proteins. These however, have the advantage that solutions can be prepared with a high degree of purity and homogeneity. For many proteins also the molecular structure and solution properties are known in detail. This may be very helpful in the interpretation of NMR data. From several of the papers in this thesis it is clear that the purity requirement with respect to transition metals is very critical. Even very small degree of metal contamination may upset the results. In all our experiments therefore careful precautions were taken to avoid metal impurities. Addition of EDTA to solutions has been avoided because EDTA-metal complexes may bind to the protein and the metal still be accessible for halide interaction (7).

The amount of protein material needed for a halide NMR experiment depends of course on the system studied and on the accuracy with which changes in the transverse relaxation rate can be monitored. In paper VI it is shown that the quadrupole coupling constant is roughly constant for different non-metal, high affinity sites on a protein. The linewidth for a bound halide could then be assumed to be proportional to the rotational correlation time if no internal motion occurs, and this is in turn proportional to the protein molecular weight. For a protein with at least one high affinity binding site 1 to 10 mg of protein should be enough to perform an NMR experiment at 0.2 M halide concentration. For studies

of halide ion interacting with a protein bound metal, 1 mg or less may be sufficient. This is much more than is normally required for a biochemical experiment but still less than required e.g. for high resolution  $^1\text{H}$  or  $^{13}\text{C}$  NMR studies of proteins.

Since the first experiments presented here were performed many advances have been made in the application of the NMR technique. For measurement of the relatively narrow signals from  $^{35}\text{Cl}$  and  $^{37}\text{Cl}$  nuclei the homogeneity and stability of the field of the first version of the wide-line instrument used were just within the limits required to obtain good reproducible data. To record spectra of solutions that contained less than 0.5 M of chloride with this instrument became very tedious. Today, signal accumulation and Fourier Transform techniques have made it possible to obtain good data in much less time and also <sup>to/</sup> record spectra of solutions with very low chloride concentration, (less than  $1 \cdot 10^{-2}\text{M}$ ).

#### Analysis of anion binding parameters.

As we will discuss below, halide NMR is a powerful method for the elucidation of the halide binding, e.g. the stoichiometry of binding, binding constants at different pH values, binding of molecules competitive to the halide ion etc..

However, halide NMR has also the potential to probe other properties of a macromolecule. In favourable cases such parameters as rotational correlation time, internal motion of the site of interaction, rates and activation energy for halide ion exchange and field



gradients at the site of binding can be obtained. The extraction of some of these parameters from relaxation is discussed in the next section.

Determination of binding constants requires a minimum of knowledge about the nature of relaxation because the change in relaxation rate will most often be linear with the concentration of bound ligands. Binding constants for halide ions are most easily obtained by an analysis of the linewidth's dependence on halide concentration at constant protein concentration. The range of binding constants accessible by such an analysis is about  $0.1 - 50 \text{ M}^{-1}$ , where the limits are determined by the halide concentration for which adequate NMR data can be obtained. Since proteins generally have several different halide binding sites a distribution of binding constants is expected. By direct analysis of the linewidth dependence it is possible to discriminate between at least two sites of different affinity if this difference is appreciable. This kind of analysis has been worked out for several of the proteins studied. For human hemoglobin A (paper I) it was found that chloride binding could be described by two binding constants at neutral pH, while at alkaline pH only one type of sites with low affinity was present. It was also shown that the affinity for chloride was different in the oxygenated and the deoxygenated form. The results are in agreement with the fact that chloride ions reduce the affinity of hemoglobin for oxygen. This implies that the functionally important chloride sites are observed. Myoglobin

was found to have only low affinity for chloride and no difference in chloride relaxation between the oxygenated and deoxygenated forms was found. For one form of hemoglobin from trout, Hb Trout I, (paper V), relaxation is to a good approximation only due to chloride sites with high affinity.  $^{35}\text{Cl}$  and  $^{127}\text{I}$  data from solutions of human serum albumin was similarly analysed (paper VIII). In this case also, the halide binding could be described by two binding constants, one type of sites with high and one with low affinity. In principle binding of any ligand, the binding of which is linked to halide binding, may be studied by halide NMR. A simple example of this is when there is direct competition between a halide ion and another ligand. NMR is then a direct and convenient method to determine the relative affinity of the halide competitive ligand. For human serum albumin (paper VIII) relative binding constants of various anions to both the strong and weak binding sites were determined. It was found that the affinity of both types of sites follows the same order as the lyotropic series, i.e. the relative ability of an ion to stabilize or destabilize a protein. These observations imply that not only electrostatic forces but also dispersive forces are important for the anion binding. The difference in affinity of binding sites suggests that suitable arrangements of nonpolar and positive amino acids in rigid cavities favour strong binding of halogen ions. In paper VI the binding of the large complex anions  $\text{Pt}(\text{CN})_4^{2-}$  and  $\text{Au}(\text{CN})_2^-$  to different proteins was studied. These ions are highly



polarizable and compete effectively with  $\text{Cl}^-$  and  $\text{Br}^-$ . As  $^{79}\text{Br}$ ,  $^{81}\text{Br}$  and  $^{127}\text{I}$  have higher NMR sensitivity than  $^{35}\text{Cl}$  and  $^{37}\text{Cl}$  it would seem to be more favourable to use these nuclei for this kind of study. However, in paper VI it is shown that the relative contribution to the linewidth from high affinity sites for Br and I is less than for Cl. This is partly due to higher saturation of weak binding sites for Br and I relative to Cl at comparable salt concentrations. It may also be due to a smaller difference in relaxation rates between the two types of sites in the case of Br and I. Hence, for a study of high affinity halide sites, which most often constitute the specific and functionally important anion binding sites, the use of chloride NMR is advantageous.

As the NMR experiments are run at halide concentrations exceeding the protein concentration by several orders of magnitude it is not possible to obtain the number of halides bound from concentration dependence data. However, it is often possible to find small molecules that are specifically bound and able to displace the halide ions stoichiometrically from one type of sites. Often these specifically bound molecules are also functionally important, e.g. enzyme substrates or inhibitors, coenzymes or effector molecules. These kinds of studies not only provide information on the number of bound halide ions and competitive ligands and possibly the position of binding but also the contribution of these sites to the total relaxation rates. In paper V

the stoichiometry of binding of the effector molecule IHP to hemoglobin is determined. In paper IV it is shown that alkaline phosphatase binds one orthophosphate per enzyme molecule. In paper VI the stoichiometric binding of the coenzyme NADH to alcohol dehydrogenase is followed by  $^{81}\text{Br}$  NMR and in paper VIII it is shown that about 9 molecules of the amphiphile sodium dodecylsulphate (SDS) bind to the high affinity chloride sites. In the latter case SDS was also used to block the high affinity sites in order to be able to study the weak sites independently. The functional properties of many proteins are regulated by specific effector molecules. The site of binding for these may be separated from the active site and the interaction mediated by changes in conformation of the protein framework, through hydrogen bonds, salt bridges etc.. Few enzymes are known to be regulated allosterically by halide ions. However, if the regulating molecule is a small organic anion the binding site may be expected to interact also with halide ions, although with considerably lower affinity. Halide NMR offers a suitable method to probe these sites without significant alterations in the functional properties of the enzyme. The  $^{35}\text{Cl}$  studies of various hemoglobins may be regarded as an example of such probing into a regulatory site. The observed difference in chloride binding between the liganded and the unliganded forms shows that at least some of the regulatory sites in hemoglobin are being probed. We have also shown (papers I and V) that specifically bound regulatory molecules like ATP and IHP are competitive with chloride ions. The fact that



we observe differential binding of chloride to the oxy and deoxy forms in the presence of saturating amounts of these molecules indicates however that the central cavity between the two beta-chains, where ATP, IHP etc. are known to bind, is not the only anion binding site linked to oxygen binding.

Thus far we have only considered applications of halide NMR to study native protein <sup>binding/</sup>anion sites. In fact the first use of halide NMR was applied to systems where halide binding sites were created by irreversible binding of mercury ions to exposed sulfhydryl groups. The interaction between chloride and the protein bound metal give rise to efficient relaxation and thus large linewidth enhancements. We have made use of this analytic method in two different studies. The specific problems encountered with halide binding to protein metal complexes will be discussed in the next section. In paper X we have used the same method as Stengle and Baldeschwieler to determine the number of sulfhydryl groups in erythrocrucorin. Titration of the protein with  $\text{HgCl}_2$  monitoring  $^{35}\text{Cl}$  linewidth gave a sharp endpoint. The change is a manifestation of the different effect that sulfhydryl bound mercury and non-specific mercury ions have on chloride relaxation. This technique has proved to be a good complement to the commonly used optical and chemical methods. In paper IV we have exploited the same method to determine the number of Zn atoms in native alkaline phosphatase. Also here we obtain an endpoint in the titration of the apo-enzyme with  $\text{Zn}^{2+}$ . In contrast to the mercury titration of

sulfhydryl groups where the metal have large effects on  $^{35}\text{Cl}$  relaxation almost no line broadening was observed for the first two  $\text{Zn}^{2+}$  added. We conclude that the first two metal ions, which are necessary for full enzyme activity, are not involved in interaction with chloride. Further addition of  $\text{Zn}^{2+}$  to the protein solution gave large signal broadening, indicating that these ions are bound on the protein surface and accessible for chloride. We have further studied the temperature dependence of  $^{35}\text{Cl}$  linewidth at different ratios of Zn to protein (unpublished) and found a decrease with increasing temperature at a Zn : protein ratio less than two. Thus there is no evidence that at low Zn content the relaxation in solution should be limited by slow exchange between protein and bulk solution.

#### Analysis of intrinsic relaxation parameters.

Interaction between proteins and anions can take place in several ways which are not easily distinguished by conventional methods. It might be expected that NMR would offer new ways for characterizing protein anion interaction. In particular one might expect higher halide relaxation rate for metallic sites. High field gradients at the nucleus due to first sphere coordination and a larger covalent contribution to the ion binding could be such as to lead to markedly higher relaxation rates than those of positively charged sidegroups on the protein. Furthermore halides coordinated to metals may for the same reason very well have slower exchange rates than those bound otherwise. These conditions



together make it likely that fast exchange in the NMR sense may not be fulfilled for metal coordinated halides on proteins. In line with these considerations, methods for discrimination between metal coordinated and general anion binding were proposed in paper II. A more general survey of the relaxation properties of different proteins was undertaken in paper VI. An analysis of relaxation data from our own work and that published by others gave support to the idea that metal coordination should give rise to considerably higher quadrupole coupling constants. This study was mainly concerned with  $^{35}\text{Cl}$ . For Br and I the intrinsic relaxation time is generally shorter due to the greater quadrupole moment and the exchange time may be longer than for chloride. Slow exchange conditions even for non-metal sites are then not unexpected for these nuclei. We also found that for high affinity non-metal chloride sites, the quadrupole coupling constant was very similar in magnitude for all proteins covered in the study. The implications of this for the binding site structure and the mode of anion binding is further discussed in paper VI.

#### Assignment of anion binding sites.

At present analysis of the halide NMR parameters offers a limited possibility for the assignment of halide binding sites. To obtain further insight into what constitutes an anion binding site we have to rely on more conventional chemical methods or a combination of these and the specific information that the NMR

method offers. The use of specific protein ligands able to displace halides has already been mentioned. Furthermore, as complex anions such as  $\text{Au}(\text{CN})_2^-$  and  $\text{Pt}(\text{CN})_4^{2-}$  are used as heavy atom derivatives in X-ray studies of proteins, they permit correlation of information from crystal structure determination and halide competition studies (paper VI).

pH studies of halide relaxation may be helpful for determining the relative contributions of differently titrating amino acids to the anion binding site.

Such studies also permit correlation with pH studies of other functional properties of a protein, e.g. the Bohr effect in hemoglobin (papers I, III and VII).

Another way to find out the position of halide sites is offered by chemical modification or by use of mutant forms of a protein. Paper VII presents a study of hemoglobin where the terminal amino acids were removed by enzymatic hydrolysis, and paper III is a study of the mutant form Hb-Abruzzo in which His  $\beta$ 143 is replaced by an arginine residue. Comparison of the pH profiles of the  $^{35}\text{Cl}$  linewidths with those of normal hemoglobin permitted identification of two different chloride binding sites. In a succeeding paper (IX) we analysed in a similar manner three other forms of hemoglobin which had structural modifications in the proposed chloride binding regions. The results further supported the assignments suggested previously.

If a change in the relaxation rate is observed on protein modification or on binding of a specific ligand,



this does not necessarily mean that a halide site is created or deleted. It could also be caused by a change in intrinsic relaxation due to alterations in the dynamics and magnitude of the field gradient at the site of binding (8).

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## References

1. Lindman, B. and Forsén, S.: Cl, Br and I NMR. Physicochemical and Biological Applications. Springer Verlag, Berlin 1976.  
Forsén, S. and Lindman, B.: Chemistry in Britain 14 29 (1978).  
Lindman, B. and Forsén, S.: Chap. 6, 7 and 13 in: NMR and the Periodic Table. (Ed. by Harris, R. and Mann, B.) Academic Press, (in print).
2. Stengle, T.R. and Baldeschwieler, J.D.: Proc. Natl. Acad. Sci. U.S.A. 55 1020 (1966).
3. Ward, R.L.: Biochemistry 8 1879 (1969).
4. Zeppezauer, M., Lindman, B., Forsén, S. and Lindqvist, I.: Biochem. Biophys. Res. Commun. 37 137 (1969).
5. Hubbard, P.S.: J. Chem. Phys. 53 985 (1970).
6. Bull, T.E.: J. Magn. Resonance 8 344 (1972).
7. Andrasko, J. and Forsén, S.: Chem. Scr. 6 163 (1974).
8. Bull, T.E., Lindman, B. and Reimarsson, P.: Arch. Biochem. Biophys. 176 389 (1976).  
Bull, T.E., Norne, J-E., Reimarsson, P. and Lindman, B.: (submitted for publication).











